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Urea and glucose modulation during freezing exposure in three temperate frogs reveals specific targets in relation to climate

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ABSTRACT

Amphibian diversity is most prominent in the warm and humid tropical and subtropical regions across the globe. Nonetheless, amphibians also inhabit high-altitude tropical mountains and regions at medium and high latitudes, exposing them to subzero temperatures and requiring behavioural or physiological adaptations to endure freezing events. While freeze tolerance has been predominantly reported in high-latitude zones where species endure prolonged freezing (several weeks or months), less is known about mid-latitudes amphibians exposed to occasional subzero temperatures. In this study, we employed a controlled ecological protocol, subjecting three frog species from the Iberian Peninsula (*Rana parvipalmata*, *Epidalea calamita*, and *Pelobates cultripes*) to a 2-h exposure to temperatures of −2 °C to investigate the accumulation of urea and glucose as physiological mechanisms associated with survival at freezing temperatures. Our results revealed a moderate response in the production of cryoprotectant metabolites under experimental freezing conditions, particularly urea, with notable findings in *R. parvipalmata* and *E. calamita* and no response in *P. cultripes*. However, no significant alterations in glucose concentrations were observed in any of the studied frog species. This relatively weak freezing tolerance response differs from the strong response exhibited by amphibians inhabiting high latitudes and enduring prolonged freezing conditions, suggesting potential reliance on behavioural adaptations to cope with occasional freezing episodes.

1. Introduction

Amphibians inhabiting cold environments with freezing temperatures have attracted considerable scientific interest due to their adaptations to thrive in subzero conditions. (e.g., Storey, 1984; Costanzo et al., 1993a; Larson et al., 2014; Voituron et al., 2009; Storey and Storey, 2017). In many cases, medium and high-latitude frog species successfully overwinter by seeking refuge in microhabitats that prevent freezing, such as water bodies or ground hibernacula (Tattersall and Ultsch, 2008; Sinsch and Leskovar, 2011, Borzée et al., 2018) against extreme climatic events (Navas, 1996, 1999). Nevertheless, a key aspect of their capacity to withstand subzero temperatures is their physiological adaptability to function at low temperatures and potentially tolerate freezing events (Carvajalino-Fernández et al., 2011).

Freeze tolerance has been predominantly documented in high-latitude regions, where species must endure prolonged freezing during winter (several weeks or months). Examples of such species are concentrated in Northern America and the northern Paleartic, including *Pseudacris crucifer*, *Pseudacris triseriata, Rana arvalis*, and *Lithobates sylvaticus* (Storey and Storey, 1992; Edwards et al., 2000; Higgins and Swanson, 2013; do Amaral et al., 2018; Shekhovtsov et al., 2022). These frogs employ complex physiological mechanisms, including the modulation of antioxidant enzymes, redox factors, nucleating and cryoprotective proteins, and cryoprotectants like glucose, glycerol, and urea, to endure freezing (Giraud-Billoud et al., 2019; Gupta et al., 2020; Shekhovtsov et al., 2022). Plasma glucose levels, for instance, can increase dramatically during freezing conditions (Storey and Storey, 1986). These adaptations serve to prevent intracellular water loss due to

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osmotic pressure from extracellular ice crystals, stabilize membrane structures (Storey and Storey, 1992), and enhance post-freezing survival (Storey and Storey, 1996). These mechanisms function in an orchestrated manner, allowing selective freezing of tissues compatible with the animal's survival. (Storey and Storey, 1988; Storey, 1990; Costanzo et al., 1993b).

Urea accumulation has also been documented in amphibians exposed to freezing temperatures. This strategy is of paramount importance for cryoprotection, maintenance of osmotic balance, preservation of cell viability, and subsequent recovery of organisms during the thawing process (Costanzo and Lee, 2005; Costanzo et al., 2013). Moreover, urea exhibits a continuous production pathway stemming from protein degradation. Additionally, its cryoprotective role has been established in scenarios where the role of glucose is either nonexistent or limited (Costanzo et al., 2014; Larson et al., 2014).

However, not all species exposed to subzero conditions develop freezing tolerance (Voituron et al., 2009), as some utilize milder microhabitats, and others exhibit population-specific adaptive variations (e.g., Costanzo et al., 2013). Limited knowledge exists regarding the freezing adaptations of amphibians exposed to occasional subzero temperatures during winter at subtropical and medium latitude regions (de Amaral et al., 2022, 2023), as well as in high-altitude tropical mountain environments (Carvajalino-Fernández et al., 2021). The latter frost boundary is situated at altitudes exceeding 3500 m in the tropical Andes, characterized by recurrent diurnal freezing spells during the night and subsequent thawing during the day (Sarmiento, 1986; Carvajalino-Fernández et al., 2011; Reider et al., 2021).

This study aims to investigate the physiological responses to freezing temperatures in three European amphibian species from different lineages. The Galician common frog (*Rana parvipalmata*), formerly part of the European common frog (*Rana temporaria*) complex (Dufresnes et al., 2020), is endemic to the northwest Iberian Peninsula, and inhabits a wide range of elevations in northern Spain (AmphibiaWeb, 2022a). The Natterjack toad (*Epidalea calamita*), has a distribution spanning from the southwestern Iberian Peninsula to northern Europe, Estonia, and east to Belarus, at elevations up to 2500 m at the Iberian Peninsula (AmphibiaWeb, 2021). The Western Spadefoot, (*Pelobates cultripes*), is distributed across the Iberian Peninsula and southern France at low elevations not exceeding 1770 m in Spain (Barbadillo, 1987; Lizana, 1997; AmphibiaWeb, 2022b). Despite populations of these species occasionally experiencing subzero temperatures (e.g., Gutiérrez-Pesquera et al., 2022 for *R. parvipalmata* populations), little is known about their freezing tolerance and cryoprotective mechanisms during freezing conditions. Moreover, previous research on the sister taxon of *R. parvipalmata*, *R. temporaria*, has primarily focused on glucose and glycerol as cryoprotectants during winter, indicating limited substrate production and storage capacity (Pasanen and Karhapää, 1997). To explore the physiological diversity in responses to short freezing events, we aim to determine urea and glucose levels in different organs of *R. parvipalmata*, *P. cultripes* and *E. calamita* during a 2-h experimental freezing exposure, simulating ecologically relevant freezing risks present in their natural environments.

2. Methods

2.1. Field collections, environmental conditions

We collected adults of the studied frog species in two different regions in the Iberian Peninsula. *Epidalea calamita* (n = 7) and *Pelobates cultripes* (n = 19) were sampled at the shoreline of Laguna de San Lázaro (N37.217,166° W6.291395°, 20 m.a.s.l.) during rainfall nights between November and December of 2015. At this sampling location, both *Epidalea calamita* and *Pelobates cultripes* are very rarely exposed to subzero temperatures. The northern and high elevation ranges distribution of *E. calamita* can imply exposures to air freezing temperatures although some indications suggest the common use of hibernation retreats by burrowing in sands (Beebee, 1983). Body temperatures in an Iberic continental climate population reached +0.3 °C during winter, demonstrating behavioural avoidance of more extreme ambient temperature, with around forty days with temperatures below 0 °C, by choosing moist and temperature-buffered microhabitats (Leskovar et al., 2006; Oromí et al., 2010). Although empirical evidence of body temperatures in *P. cultripes* are available, their southern Mediterranean range and burrowing abilities also suggest the possibility of environmental buffering of eventual subzero temperatures. Additionally, both natterjacks and *P. cultripes* tadpoles and juveniles have around zero CT_{min} values, ranging between +0.6 °C to -1 °C (Gutiérrez-Pesquera et al., 2016, M. Tejedo, and L. M. Gutiérrez-Pesquera, unpublished data). *Rana parvipalmata* (n = 28) were collected in a mountain wetland at Vega de Candioches (Castile and León: N42.99,883° W5.92,076°, 1687 m.a.s.l.) from mid-October to late-November of 2015. Common frogs in northwestern Iberia (*Rana parvipalmata* and *Rana temporaria*) are highly exposed to short periods of freezing (i.e., subzero temperatures: A.G. Nicieza and D. Álvarez, unpublished data) during the time prior to hibernation and at the start of breeding in mountain areas (due to snow storms), or by mid-winter in mid- and low-elevation areas where frogs do not hibernate and can be exposed to temperatures below zero due to rapid cooling events during the breeding season, which can occur at any point from early September to late February in these areas (see Gutiérrez-Pesquera et al., 2022). Enríquez-Urzelai et al. (2020) reported average CT_{min} of around −1.65 and −1.95 °C for highland and lowland populations, whereas Gutiérrez-Pesquera et al. (2022) found CT_{min} averaging from −0.9 to −2.3 °C for a set of 11 populations distributed across an elevational gradient of 1800 m. Although there are no previous studies on the hibernating habits of *Rana parvipalmata* and *Rana temporaria* populations in northwestern Iberia, ground hibernacula are the only option for many mountain populations where permanent water bodies are absent. We cannot discard the use streams in wetlands like Candioches where water can flow beneath the snow cover in winter. In any case, these frogs can avoid freezing temperatures over the hibernation period by seeking refuge either in flowing waters or in ground cavities.

After collection, frogs were carried out to the Laboratorio de Camaras Climáticas (LCM-EBD) in the Estación Biologica Doñana – EBD-CSIC, Sevilla Spain, and maintained in isolated cages at a temperature of 17 °C (range 16-18 °C), exposed to a photoperiod of 12:12 (L:D), and fed with earthworms and water *ad libitum* for one week. *P. cultripes* and *E. calamita* toads were collected under permit reference code: SGYB/FOA/AFR/CFS September 6, 2012, CMAOT of JJAA. *R. parvipalmata* individuals were collected under license number: EP/CYL/725/2015.

2.2. Freezing protocol, survival test and recovery capacity

Before freezing assays, both control and experimental frogs were fasted 24 h to avoid bias in biochemical levels due to feeding. They were also acclimated to cold by placing them directly in a refrigerator at 7 °C (range 5 °C-8°C) for 1 h, simulating a temperature drop from 17 °C to 7 °C. Afterwards, experimental frogs were randomly split into small groups of three individuals each. These groups were placed in airtight 1000 ml top jars and submerged for 2 h in a low-temperature bath of ethanol (20 %). The temperatures inside the jar were measured using HOBO Pendant® UA-001-08, which were positioned at the bottom and in contact with frog skin. The temperature regulation of the bath was controlled using a resistor set at −2.1 °C that switched off at −1.9 °C yielding a low voltage (5 mvol) and resulting in a mean experimental temperature of mean ± SD: −2.0 °C ± 0.2 °C (Carvajalino-Fernández et al., 2021; Costanzo et al., 2013). Control individuals were also placed in 1000 ml top jars and kept in the refrigerator at 7 °C.

After the freezing trials, all control and experimental frogs were transferred within their containers to a room at 17 °C for recovery. Transferring the entire container ensured that the heating rate was not as abrupt for the animal. Survival was assessed as the fraction of

recovered individuals for the experimental frogs within 2 h after thawing. Recovery was considered complete if frogs showed regular breathing, normal posture, and jumped following light prodding. Similarly, we also estimated survival for the control group. Following the recovery assessment, the non-freezing animals: *R. parvipalmata* (n = 9); *P. cultripes* (n = 8); *E. calamita* (n = 3) and experimental animals: *R. parvipalmata* (n = 19); *P. cultripes* (n = 11); *E. calamita* (n = 4) were euthanized with liquid nitrogen and sampled. All experimental protocols were approved by CMAOT of JJAA for conducting the experiments (Ref. 12_44).

2.3. Protocol test for urea and glucose

Following confirmation of the frogs' death after euthanasia, the liver, heart and muscle (gracilis) from the right hindlimb were swiftly removed under ice. Tissue samples were then stored at −80 °C before metabolite analyses were conducted. For each organ, tissue sampleswere added to tubes and homogenized with an electric mechanical homogenizer. Subsequently, the tubes were centrifuged at 10.000 rpm for 10 min at 4 °C. All parameters were determined from the supernatant using a Clinical Chemistry Analyzer COBAS 600 (Roche Diagnostics). The biochemical tests and their corresponding methods were implemented as follows: Urea analysis was carried out using a kinetic test employing a urease enzyme, while glucose analysis was conducted utilizing the oxidase/phenol + aminophenazone method, commonly denoted as GOD/PAP. The results were shown as μmol. g^{-1}.

2.4. Statistical analysis

Data were examined previously for statistical analysis for normality and homogeneity of variance assumptions using Shapiro-Wilk test for small sample sizes (Zuur et al., 2009). We conducted a two-way analysis of variance (ANOVA), within each examined species using as fixed parameters: tissue (heart, muscle, and liver) and experimental exposure (non-freezing and freezing) and its interaction. The analysis was complemented with Sidak's multiple comparisons test. The comparison of urea and glucose concentrations between the nonfreezing and freezing within each species and tissue was analyzed by unpaired *t*-test. Comparisons between the species and the urea glucose concentrations in each tissue during the different treatments were conducted by one-way ANOVA following Tukey's multiple comparisons test. The urea and glucose concentrations in pooled tissue between species were analyzed by one-way ANOVA following Tukey's multiple comparisons test. The results were shown as mean and standard error of mean (SEM), and the differences were considered significant when $p < 0.05$. The statistical analyses were performed with GraphPad Prism® version 8.0.2. Effect sizes were estimated with Metawin 2.1 (Rosenberg et al., 2000).

3. Results

3.1. Survival and recovery from the freezing conditions

After the freezing treatment, individuals of the three species exhibited stiff and brittle limbs, solid abdomens and opaque eyes, indicating tissue freezing. Visual inspection suggested that heartbeats and respiratory activity halted in response to treatment. Recovery was considereded complete (regular breathing, normal posture, and jumped following light prodding) within 2 h after thawing for all survivors. The survival percentage of the experimental group frogs after the recovery period was 100 % in *R. parvipalmata*, and 75.0 % and 72.2 % in *E. calamita* and *P. cultripes,* respectively. All control frogs survive after the recovery period.

3.2. Cryoprotectants variation

3.2.1. Urea

Within-species variation revealed that urea concentrations varied across tissues, being greater in the liver than muscle and heart in *R. parvipalmata* ($p < 0.001$), and in liver than in the heart in *P. cultripes* ($p < 0.003$), but not for *E. calamita* (Fig. 1, Table S1 in Supplementary Material). Nevertheless, urea did not vary across freezing and non-freezing treatments and in the interaction tissue x experimental exposure for any studied species (Supplementary material S1). However, when analyzing the urea concentrations within each tissue for each species (Table 1), *Rana parvipalmata* showed a difference in heart with higher urea concentration in freezing compared to non-freezing ($t = 1.66$, $df = 19$, $p = 0.0093$; Fig. 2a). Likewise, *E. calamita* had higher urea muscle concentrations in freezing compared to non-freezing groups ($t = 0.3432$, $df = 2$, $p = 0.018$; Fig. 2b). However, *P. cultripes* did not show any significant difference in urea concentrations between freezing and nonfreezing treatments ($t = 1.709$, $df = 14$, $p = 0.109$, Table 1). When comparing urea concentrations between species for all tissues pooled, we observed that *R. parvipalmata* showed the lowest tissue concentrations, approximately half of those observed for *P. cultripes* and up to one-eighth times lower than those of *E. calamita*, for both non-freezing and freezing treatments (Supplementary material S3).

3.2.2. Glucose

Within-species variation revealed that glucose concentrations varied among tissues, being greater in the liver than in muscle and heart. Similar to urea, glucose did not vary across experimental exposures, and we did not detect a significant tissue × freezing interaction for any of the studied species (Fig. 3, Supplementary material S2). Also, when examining glucose concentrations within each tissue for each species, freezing conditions did not change glucose concentrations with respect to non-freezing in any species (Table 2). Contrary to the pattern found for urea, glucose concentrations for all tissues pooled did not vary between species (Supplementary material, Table S4).

4. Discussion

During freezing, some anurans of high latitudes display several physiological adaptations, such as an increase in the concentrations of cryoprotectant metabolites like glucose and urea (Storey, 1984; Costanzo et al., 2014). However, the mobilization of metabolites in frogs that live at mid-latitudes is still not clear. Thus, our study assessed the concentrations of urea and glucose during brief freeze exposure in three temperate species belonging to three anuran families: *Rana parvipalmata*, *Epidalea calamita*, and *Pelobates cultripes*. The ranid *R. parvipalmata* may naturally encounter long-term freezing conditions (Gutiérrez-Pesquera et al., 2022), while the studied Mediterranean populations of the pelobatid *P. cultripes* and the bufonid *E. calamita* seldom experience short episodes of subzero temperatures during their winter breeding activity. Our study revealed a moderate response in the production of cryoprotectors, particularly urea, under experimental freezing conditions for both *R. parvipalmata* and *E. calamita* in heart and muscle tissues, respectively. For *P. cultripes*, freezing conditions did not trigger an increase in muscle urea concentrations. In the case of glucose, however, no significant response was observed in any of the studied frog species. These findings indicate that the dynamics of cryoprotectant production and response to short freeze exposure differ among these temperate species, naturally exposed to contrasting cold stress, but are generally weak.

Previous studies showed that *R. temporaria* could not be considered a freezing-tolerant species. For instance, all individuals from a Danish population survived exposures to −2 °C for 1 h; however, longer exposures to −2 °C for 24 h resulted in a strong decline in survival (Voituron et al., 2009). Additionally, an Austrian population of this species did not exhibit any seasonal increase in glucose concentrations under subzero field temperatures (Ludwig et al., 2015). However, Pasanen & Karhapää (1997) reported survival rates of 100% for exposition to -2 °C for 24 h and partial survival (33–77%) for 48-h exposure periods. Our studied *R. parvipalmata*, a southernmost sister species of *R. temporaria*

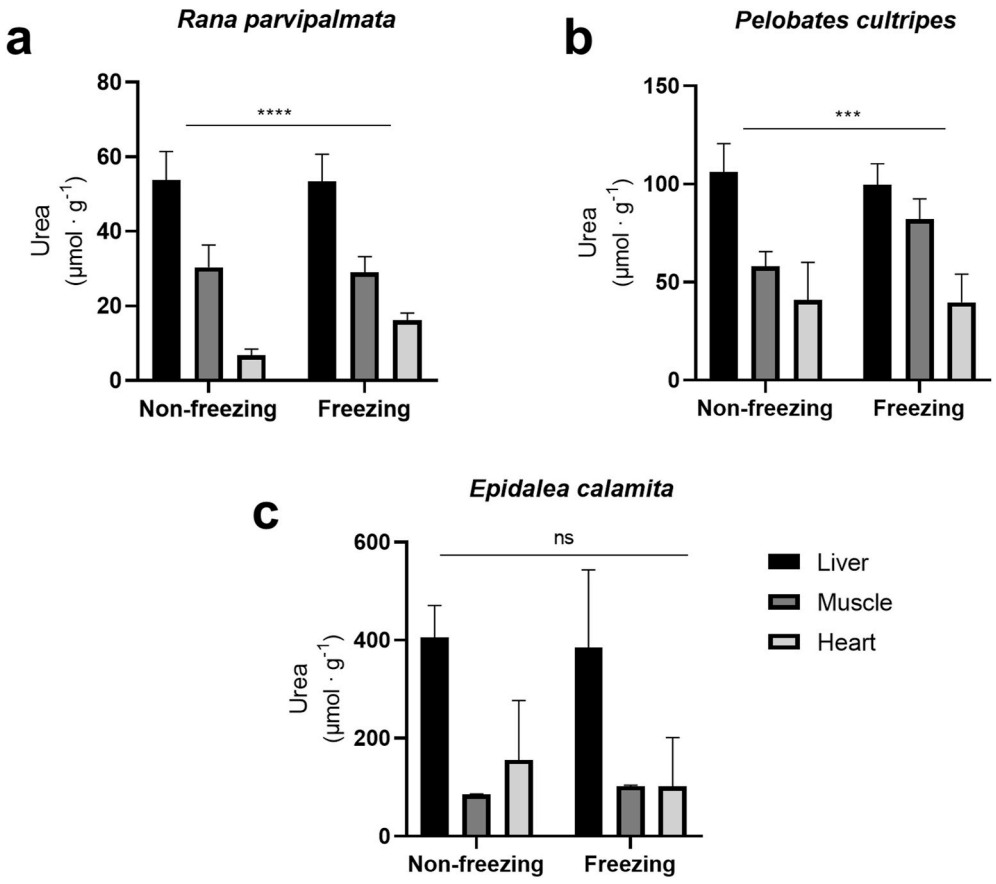

Fig. 1. Urea concentrations (mean + 1 SEM) between non-freezing and freezing in **(a)** *Rana parvipalmata*, **(b)** *Pelobates* cultripes, and **(c)** *Epidalea calamita*. (****: $p < 0.0001$, ***$p < 0.001$, ns: non-significant. Probabilities associated with species comparisons of urea concentrations of pooled tissues, see Results).

Table 1
Comparisons of urea concentrations (μmol g^{-1}) in specific tissues (liver, heart and muscle) between non-freezing and freezing treatments for the studied species: *Rana parvipalmata*, *Pelobates cultripes* and *Epidalea calamita.* Species values are expressed as the mean and respective standard error of the mean (SEM). Unpaired *t*-test was used for comparisons and the results were considered significantly different when $p \leq 0.05$ (marked in red bold).

Urea concentrations (mmol · L^{-1})															
	Liver					Heart					Muscle				
	n	Non-freezing	n	Freezing	*p*-value	n	Non-freezing	n	Freezing	*p*-value	n	Non-freezing	n	Freezing	*p*-value
Rana parvipalmata	9	50.87 ± 7.35	17	53.42 ± 7.27	0.824	7	7.38 ± 1.536	15	16.15 ± 1.934	**0.009**	8	26.58 ± 6.45	19	31.56 ± 3.831	0.497
Pelobates cultripes	5	106.2 ± 14.41	9	99.69 ± 10.67	0.723	7	22.26 ± 5.62	9	26.04 ± 5.07	0.627	8	57.95 ± 7.57	8	82.8 ± 12.42	0.109
Epidalea calamita	2	405.4 ± 65.48	4	384.8 ± 158.5	0.936	2	155.7 ± 121.2	2	101.9 ± 99.39	0.764	2	85.24 ± 1.25	2	138.9 ± 7.19	**0.018**

(Dufresnes et al., 2020, 2023), appears to exhibit an identical pattern. Similar to the findings of Voituron et al. (2009), *R. parvipalmata* exhibited full survival at −2 °C during our shorter period experimental protocol (2 h). However, it is worth noting that more intense and prolonged exposure to freezing conditions could significantly decrease its viability. The most compelling evidence supporting the weak freezing tolerance capacity of *R. parvipalmata* is the absence of an increase in glucose under freezing conditions, which is consistent with the results of Ludwig et al. (2015). However, we cannot rule out the potential of *R. parvipalmata* adults to tolerate freezing temperatures, as both *R. temporaria* tadpoles and juveniles can reach subzero values for lower critical thermal limit (CT_{min}) (Enríquez-Urzelai et al., 2020; Gutiérrez-Pesquera et al., 2022), and adults can be observed at temperatures near zero degrees either on ground surface or below the ice cover of shallow, frozen ponds, after a sudden fall in temperature during peaks of breeding activity (A.G. Nicieza and D. Alvarez, unpublished data). It should be pointed out that freezing protection in common frogs might be even more important for lowland (non-hibernating, breeding from September to February) than for mountain populations (see also Muir et al., 2014; Ludwig et al., 2015), because the former is more exposed to pond freezing during their breeding period.

Nonetheless, compelling evidence supporting the hypothesis of freezing adaptation in *R. parvipalmata* is the substantial increase in urea concentrations in heart tissue during our brief experimental exposure to

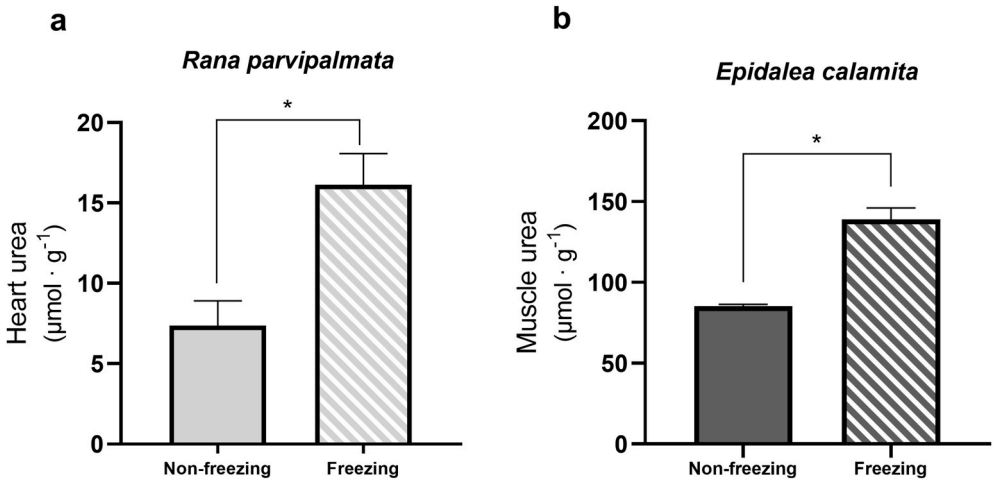

Fig. 2. Urea concentrations (mean + 1 SEM) of Non-freezing and Freezing exposures in **(a)** *Rana parvipalmata* heart tissue and **(b)** *Epidalea calamita* muscle tissue. *: $p < 0.05$, Unpaired *t*-test.

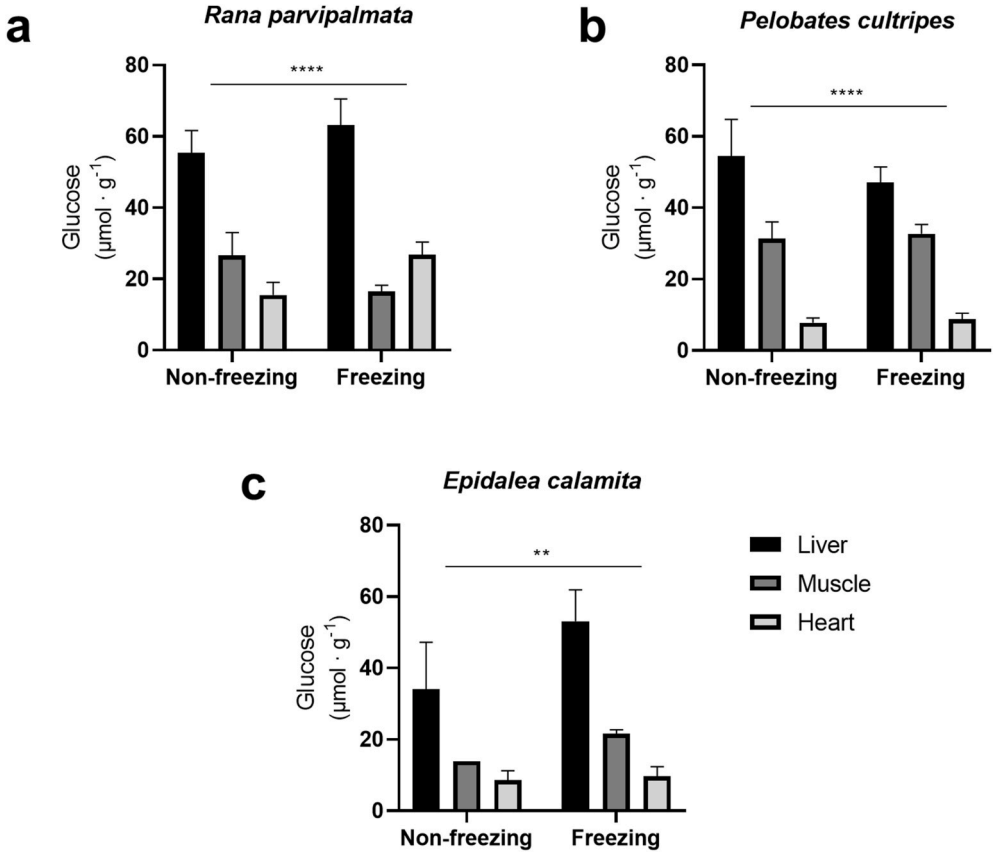

Fig. 3. Glucose concentrations (mean + 1 SEM) between non-freezing and freezing exposure in *Rana parvipalmata* **(a)**, *Pelobates* cultripes **(b)** and *Epidalea calamita* **(c)**. ****: $p < 0.0001$, **$p < 0.01$. Probabilities associated with species comparisons of urea concentrations of pooled tissues, see Results.

freezing, which contributes to tissue viability and integrity (Costanzo and Lee, 2005). The role of urea when protecting ectotherms against freezing damage is considered functionally equivalent to glycerol, an even better cryoprotectant than glucose in freeze-tolerant frogs such as *Nanorana pleskei* (Niu et al., 2018) and *L. sylvaticus* (Costanzo and Lee, 2013). Although *R. parvipalmata* appears to show a lower urea accumulation during freezing exposure compared to *N. pleskei* and *L. sylvaticus*, the magnitude of effect sizes comparing means of freezing and non-freezing groups revealed equivalent between species (d+, variance, *L. sylvaticus*: 1.226, 0.367; *N. pleskei*: 1.277, 0.241; *R. parvipalmata*, 1.269, 0.246), thus suggesting a similar freezing response for our species. This relatively lower urea concentration in *R. parvipalmata* may still provide sufficient cryoprotective benefits while avoiding issues related to the seasonal regulation of nitrogen metabolism, as demonstrated in *L. sylvaticus* (Costanzo et al., 2014). Furthermore, the presence of urea can depress the metabolism of the liver, stomach, and heart (Muir et al., 2008). Thus, the observed increase during freezing exposure in *R. parvipalmata* could be an adaptive strategy to prevent both ice injury and the osmotic dehydration of cells (Costanzo and Lee 2008). In addition, the lower urea concentration

Table 2
Comparisons of glucose concentrations (μmol g^{-1}) in specific tissues (liver, heart and muscle) between non-freezing and freezing treatments for the studied species: *Rana parvipalmata*, *Pelobates cultripes* and *Epidalea calamita.* Species values are expressed as the mean and respective standard error of the mean (SEM). Unpaired *t*-test was used for comparisons and the results were considered different when $p \leq 0.05$.

Glucose concentrations (mmol · L^{-1})														
	Liver					Heart					Muscle				
	n	Non-freezing	n	Freezing	*p*-value	n	Non-freezing	n	Freezing	*p*-value	n	Non-freezing	n	Freezing	*p*-value
Rana parvipalmata	9	55.37 ± 6.25	17	62.11 ± 6.99	0.534	6	16.18 ± 4.05	15	26.74 ± 3.65	0.113	9	16.95 ± 2.08	17	16.53 ± 1.71	0.882
Pelobates cultripes	7	54.45 ± 10.35	11	47.12 ± 4.28	0.463	8	7.74 ± 1.37	9	7.321 ± 1.1	0.814	7	31.39 ± 4.63	11	32.72 ± 2.55	0.788
Epidalea calamita	3	52.91 ± 11.6	4	64.79 ± 7.32	0.403	3	8.59 ± 2.66	4	9.68 ± 2.68	0.789	3	22.43 ± 2.19	4	25.63 ± 3.21	0.482

accumulated can help to avoid hypometabolism (Muir et al., 2008) and the possibility of a quick recovery of neurobehavioral functions following thawing or inactivity brought on by the dormancy experience (Costanzo and Lee, 2008). In a context of continuous freezing and thawing cycles, as observed in many populations of *R. parvipalmata* at mid and high elevations (Enriquez-Urzelai et al., 2018; 2020), urea may indeed play a pivotal role in the preservation of such a vital organ as the heart.

Regarding the weak responses to freezing exhibited by *E. calamita,* two factors could explain it. First, no freezing adaptations were found in four bufonids (*Anaxyurus americanus, A. woodhousii, A. cognatus,* and *B. bufo)* inhabiting northern latitudes and mountains in North America and Eurasia (Voituron et al., 2009), suggesting a possible phylogenetic constraint. Second, it has been reported that *E. calamita* toads actively burrow into moist and temperature-buffered microhabitats to avoid extreme environmental temperatures (Oromí et al., 2010; Sinsch and Leskovar, 2011), demonstrating that they prioritize a behavioural adaptation over a physiological one. However, *E. calamita* displayed a significant increase in urea concentration in muscle tissue (effect size, d+ = 4.205, var = 3.21) during freezing exposure, similar to the findings in *R. parvipalmata*, (see above), *L. sylvaticus* (Costanzo and Lee, 2014), and *N. pleskei* (Niu et al., 2018) heart tissues. Previous research has suggested that urea can play a role in preserving post-thaw tissue integrity, thereby limiting freezing injury in skeletal muscle (Costanzo and Lee, 2005). Furthermore, muscles that exhibited reduced activity after the freezing-thawing process can regain higher levels of contractile performance when pretreated with urea (Costanzo et al., 2007). Therefore, *E. calamita*, by increasing urea levels in its muscle tissue, may be conserving the integrity of its locomotion capacity while awaiting, buried, the 'warmest days' of winter for reproduction or foraging, as suggested by Salvador et al. (2021).

Pelobates cultripes did not exhibit any significant accumulation of either urea or glucose, indicating limited tolerance to cold conditions or other strategies to cope with subzero temperatures. Similarly, the related species *P. fuscus*, which inhabits much colder ranges in Eurasia (Dufresnes et al., 2019), quickly succumbs to temperatures slightly below 0 °C (Berman et al., 2019). Further studies are needed to determine if other cryoprotectants, such as glycerol increase during freezing episodes (do Amaral et al., 2018), to conclude that *P. cultripes* relies solely on behavioural adjustments to cope with cold spells.

Our data indicate a modulation of urea associated with the protection of heart integrity during constant freezing and thawing events in *R. parvipalmata* in the northern mountain region of the Iberian Peninsula. Similarly, during the mild Mediterranean winter, the species *E. calamita* may be utilizing urea mobilization in its muscle to preserve locomotion capacity during freezing events, while waiting for rainy nights with 'warmer' temperatures during its winter breeding season. It is worth remembering that our protocol of 2 h of freezing exposition reflects a very conservative representation of the freezing risk these species could face in the field. The response patterns of species inhabiting habitats with sporadic subzero temperature exposures remain unclear in the literature (Carvajalino-Fernández et al., 2021; Reider et al., 2021; de Amaral et al., 2023), emphasizing the need for new studies involving these species and other ectotherms with freeze tolerance capabilities, when exposed to episodic cold spells typical of medium latitudes and high mountains in the tropics (Carvajalino-Fernández et al., 2011, 2021; Costanzo and Lee, 2013; Reider et al., 2021).

5. Conclusions

This study offers novel insights into the variability of the freeze physiological response in amphibians, emphasizing the importance of examining the stress associated with freezing exposure within the ecological context of the respective population and species. This approach considers their physiological constraints influenced by natural history and phylogenetic relationships. Undoubtedly, further investigations involving species experiencing brief freezing periods are necessary for elucidating the underlying physiological patterns and understand the physiological adaptations to subzero exposure temperatures in amphibians.

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CRediT authorship contribution statement

Marjoriane de Amaral: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Juan Manuel Carvajalino-Fernández:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Alfredo G. Nicieza:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Miguel Tejedo:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

Data availability

The data that support the findings of this study are available upon request to the corresponding author or online at https://osf.io/ndpvm/?view_only=7fba9f8afbba4b669613ae5a31d5a4e2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2024.103854.

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